Hepatoprotective and Antioxidant Effects of Ethanol Extract from *Phellinus merrillii* on Carbon Tetrachloride-Induced Liver Damage

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Abstract: In the present study, we investigated the hepatoprotective and antioxidant capacities of ethanol extract of *Phellinus merrillii* (PM) on carbon tetrachloride-induced hepatotoxicity. In high-performance liquid chromatography (HPLC) analysis, the fingerprint chromatogram of PM was established. Both hispolon and PM showed a similar peak at the retention time of 6 min. This implied that PM did contain the active ingredient of hispolon. Treatment with PM (0.5, 1 and 2 g/kg) prior to the administration of carbon tetrachloride (1.5 ml/kg in olive oil, 20%) significantly prevented the increased serum alanine aminotransferase (s-GOT) and serum aspartate aminotransferase (s-GPT) in a dose-dependent manner. We also found that the incidences of ballooning degeneration, necrosis and portal triaditis were lowered in the group pretreated with PM. Carbon tetrachloride induces up-regulation of antioxidant enzymes, including superoxide dismutase (SOD) (36.6%), catalase (58.8%) and glutathione peroxidase (GPx) (64.7%) in the liver. Pretreatment with PM significantly reduced all these antioxidant enzyme activities. Therefore, we verified that ethanol extract of PM has the hepatoprotective and antioxidant capacities on rats.

Keywords: *Phellinus merrillii*; Carbon Tetrachloride; Hepatoprotective Effect; Antioxidant; High-Performance Liquid Chromatography.
Introduction

Carbon tetrachloride (CCl₄) is an extensively used xenobiotic to induce lipid peroxidation and hepatoxicity. CCl₄ is metabolized by cytochrome P450 2E1 (CYP2E1) to the trichloromethyl radical (CCl₃•), which would initiate free radical-mediated lipid peroxidation leading to the accumulation of lipid-derived oxidation products that cause liver injury (Poli et al., 1987; Recknagel et al., 1989). Polyunsaturated fatty acids (PUFAs) in membrane lipids are especially susceptible to free radical-initiated peroxidation (Svingen et al., 1979). PUFAs in phospholipids of the endoplasmic reticulum were decreased following in vivo CCl₄ administration (James et al., 1982). A number of investigators have previously demonstrated that antioxidants would prevent CCl₄ toxicity, particularly hepatotoxicity, by inhibiting lipid peroxidation (Teselkin et al., 2000), suppressing s-GOT and s-GPT activities (Ohta et al., 1998; Lin et al., 2000), and increasing the activities of antioxidant enzymes (Kumaravelu et al., 1995). This experiment was also to explore the defense mechanism involving the antioxidant enzymes, including SOD, catalase, and GPx, which convert active oxygen to non-toxic compounds.

Silymarin is a group of flavones extracted from Silubum marianum L. Gearth, and is a strong antioxidant (Mitchell et al., 1973; Hjelle and Klaassen, 1984). Silymarin is an effective agent for liver protection and liver cell regeneration. In this study, silymarin was used as a positive control drug.

Macrofungi was commonly used as a nutrition supplement to a variety of diseases in Asia (Jong and Birmingham, 1992; Chen et al., 2006b). In Taiwan, several different species of Phellinus were widely applied for anticancer and also for antioxidant purposes. The anti-tumor activity of Phellinus linteus has been demonstrated in several studies (Han et al., 1999; Lin et al., 2003; Kim et al., 2003; Li et al., 2004; Bae et al., 2005). Also, there were several reports about the antioxidant effects of Phellinus. The basidiocarps, isolated from the methanolic extract of Phellinus linteus, have shown antioxidative effect (Chung et al., 1998; Sohn and Nam, 2001). Studies indicated that Phellinus linteus could protect primary cultured rat hepatocytes against hepatotoxins (Kim et al., 2004). Also, Phellinus rimosus (Berk) Pilat possess antioxidant and antihepatotoxic activities (Ajith and Janardhanan, 2002). Hispolon, a yellow pigment first found in Inonotus hispidus in 1996 (Ali et al., 1996b), was isolated from the fungus Phellinus igniarius (Mo et al., 2004). Hispolon had been reported to exhibit apoptosis effect on human epidermoid KB cells (Ali et al., 1996a) and antiviral activities (Awadh et al., 2003). Hispolon also inhibit chemiluminescence response of human mononuclear cells and suppress mitogen-induced proliferation of spleen lymphocytes of mice (Ali et al., 1996a). In a previous work, we showed (unpublished data) that PM displayed antioxidant activities in a series of in vitro tests, such as DPPH (1, 1-diphenyl-2-picrylhydrazyl) staining and reducing power method. The purpose of this experiment was to evaluate the hepatoprotective and antioxidant effects of the ethanol extract of PM on carbon tetrachloride-induced liver damage.
Materials and Methods

Chemicals

CCl₄ was purchased from Merck (Germany). Silymarin was purchased from Sigma Chemical Co. (Steinheim, Germany). Formalin was purchased from Nihon Shiyaku Industries (Japan). Ethyl ether was obtained from Shimakyu’s Pure Chemicals (Osaka, Japan). Biochemical assay kits (ALT kits: Art.J16651; AST kits: Art.As483; Protein kits: Art.K83001; SOD kits: ArtORD04; GPx kits: Art.J68131) were obtained from Randox Laboratories Ltd. (UK). Hispolon was purchased from Bjym Pharm. & Chem. Co. Ltd. (Beijing, China).

Materials

Fresh fruiting bodies of PM were purchased from Ji-Pin mushroom store (Nantou, Taiwan). PM was identified by Dr. Yu-Cheng Dai from the Institute of Applied Ecology, Chinese Academy of Science, China and Dr. Sheng-Hua Wu from the Department of Botany, National Museum of Natural Science, Taiwan.

Extraction of Phellinus merrillii

PM, 1.5 kg was soaked with 70% ethanol at room temperature. The samples were filtered with filter paper (Advantec No. 1, Japan), the residue was further extracted under the same conditions three times. The filtrates collected from these separate extractions were combined and evaporated to dryness under vacuum. The yield obtained for PM is 4% (60 g).

Compositional Analysis of Hispolon and PM by HPLC

HPLC was conducted to analyze both the standard (hispolon) and PM according to Chen et al. (2006a). The purity of standard (hispolon) was found to be more than 95% based on reversed phase HPLC analysis (Instrument: Waters 2695; Column: Cosmosil 5C18-AR-II Waters, 4.6’150 mm, 5 µ; Mobile phase: AcCN:H₂O (50:50); Flow rate: 0.5 ml/min; PDA: 200~350 nm).

Animal Treatment

Male Sprague-Dawley (SD) rats (200–250 g) were obtained from the Laboratory Animal Breeding and Research Center of the National Science Council (Taipei, Taiwan). Rats were housed individually and maintained under temperature control of 21 ± 1°C. Animals were kept on a 12-hour light/dark cycle, with diet and water supplied ad libitum.
were randomly distributed into 6 groups of 8 animals. Rats in the normal control and CCl₄ groups were orally administered with distilled water. Rats in the positive control group were administered with silymarin (200 mg/kg in 1% carboxymethyl cellulose). Low (0.5 g/kg), medium (1 g/kg) and high (2 g/kg) doses of PM were administered by gavage in CCl₄ groups. After 1 hour, rats were i.p. injected with CCl₄ (1.5 ml/kg in olive oil, 20%), and olive oil (1.5 ml/kg) for the normal control. Twenty four hours after the injection of CCl₄ or olive oil, animals were anesthetized with ethyl ether and the blood was collected through the neck arteries.

**Assessment of Liver Functions**

The blood was centrifuged at 3,000 rpm (Beckman GS-6R, Germany) at 4ºC for 30 min to separate serum. Serum alanine and aspartate aminotransferase were therefore analyzed. Liver tissues collected from the animals were stored at 10% formalin for their histopathological studies. Also, liver tissue was kept under −80ºC for further analysis of their enzyme activities. The biochemical parameters were analyzed by using clinical test kits (Roche, Germany) spectrophotometrically (Roche Cobas Mira Plus, Germany).

**Histopathological Examination**

Hepatic tissues of the animals were collected from the same lobe of the livers and trimmed to an approximately 2 mm thickness. Then, tissues were placed in 10% of neutral buffered formalin for histopathological studies. For histopathological examination, the formalin fixed liver was embedded in paraffin, cut into 4–5 µm thick sections, stained with hematoxylin-eosin, and observed under a photomicroscope.

**Antioxidant Enzyme Activity Measurements**

The following biochemical parameters were analyzed to check the hepatoprotective activity of PM by the methods given below. SOD activity was determined by monitoring the inhibition of cytochrome C reduction at 550 nm using xanthine and a xanthine oxidase system. One SOD unit was defined as the amount of enzyme that inhibited cytochrome C reduction by 50% (Lee and Yu, 1990). GPx activity was determined by the method of Rotruck *et al.*, (1973). SOD was estimated by the method of Kakkar *et al.*, (1984). Catalase activity was measured by the method of Aebi (1984). Reaction mixture (2 ml), which contained 0.01 ml of liver homogenate, 0.09 ml of 1% Triton X-100 and 1.9 ml of 20 mM of phosphate buffer, was added to a crystal cuvette containing 1 ml of 0.03 M H₂O₂. Changes in absorbance were read at 240 nm for 1 min. Using the reaction time interval (Δt) of absorbance (A1 and A2), the rate constant (K) was calculated following the equation $K = (2.3/Δt) \log (A1/A2)$. The specific activity of the enzyme was expressed as K/mg protein.
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Total Protein Assay

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Statistical Analysis

All experiments were repeated at least three times. Means ± SEM were calculated for each group and student’s t test (unpaired) was used to calculate statistical significance. Differences were considered statistically significant when p < 0.05.

Results

Compositional Analysis of Hispolon and PM by HPLC

We analyzed the authentic compound, hispolon and the composition of PM by HPLC. The HPLC fingerprint of PM is shown in Fig. 1. Both standard and PM showed similar peak at the retention time of 6 min. The chromatogram indicated that PM did contain the active ingredient hispolon and the HPLC fingerprint of PM can provide the chemical basis for future repetitive trials.

Effects of Phellinus merrillii on CCl₄-Induced Acute Hepatotoxicity of Rats

CCl₄ significantly increased s-GOT and s-GPT in rats (Figs. 2 and 3). However, the elevated magnitudes of CCl₄-induced s-GOT and s-GPT were markedly reduced by pretreatment with PM (0.5, 1, and 2 g/kg) in a dose-dependent manner. PM could protect the liver from CCl₄-induced liver damage.

Figure 1. HPLC chromatograms of PM and hispolon (standard). Instrument: Waters 2695; Column: Cosmosil 5C₁₈-AR-II waters, 4.6’150 mm, 5 µl; Mobile phase: AcCN:H₂O (50:50); Flow rate: 0.5 ml/min; PDA: 200~350 nm. Retention time: 6.01; sample (10 mg/10 ml MeOH); hispolon (2 mg/10 ml MeOH); Inj. vol. 10 µl.
Histopathology of the Liver

Figure 4 showed that CCl₄ could induce histological changes, including increased degeneration, necrosis, hepatitis and portal triaditis. All rats except those in the control group exhibited the ballooning degeneration in the centrolobular zone and the necrosis of hepatocytes (Fig. 4). The CCl₄-induced damage suffered more severely than those pretreated with PM groups. This finding was consistent with the levels of the enzymes markers.
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**Figure 4.** Photomicrograph of liver section from rats and stained with hematoxylin-eosin. (A) control; (B) CCl$_4$ only (1.5 ml/kg); (C) Silymarin (200 mg/kg) + CCl$_4$ (1.5 ml/kg); (D) PM (0.5 g/kg) + CCl$_4$ (1.5 ml/kg); (E) PM (1.0 g/kg) + CCl$_4$ (1.5 ml/kg); (F) PM (2.0 g/kg) + CCl$_4$ (1.5 ml/kg).

**Effects of Phellinus merrillii on the Activities of Antioxidant Enzymes**

The activities of antioxidant enzymes (SOD, catalase and GPx) in the liver of the animals of CCl$_4$ groups were substantially higher than those in the control group (Figs. 5–7). PM reduced these enzyme activities in a dose-dependent manner.

**Discussion**

This work demonstrated that the ethanol extracts of PM significantly attenuated CCl$_4$-induced hepatotoxicity of the rats. CCl$_4$ is a highly hepatotoxic compound. The mechanism of
Figure 5. Effect of ethanol extract of *Phellinus merrillii* on the activities of SOD in rats 24 hours after CCl₄ treatment. Each value is a mean ± SEM. *p < 0.05, compared to the control group, **p < 0.01, ***p < 0.001 compared to the CCl₄ group.

Figure 6. Effect of ethanol extract of *Phellinus merrillii* on the activities of catalase in rats 24 hours after CCl₄ treatment. Each value is a mean ± SEM. #p < 0.05, compared to the control group, *p < 0.05 compared to the CCl₄ group.

Figure 7. Effect of ethanol extract of *Phellinus merrillii* on the activities of GPx in rats 24 hours after CCl₄ treatment. Each value is a mean ± SEM. 5p < 0.05, compared to the control group, 6p < 0.05, 7p < 0.01, 8p < 0.001 compared to the CCl₄ group.
CCL₄ induced liver damage involved the biotransformation of CCL₄ by the cytochrome P450 (CYPs) system into a trichloromethyl free radical (CCl₃•) which causes lipid peroxidation, disrupts Ca²⁺ homeostasis, and eventually kills cells (Gonzalez, 1988; Recknagel et al., 1989; Farombi, 2000). Administration of CCL₄ to rats markedly increases s-GOT and s-GPT levels (Jin et al., 2005). This increase commonly reflects the severity of liver injury (Bhathal et al., 1983; Lin et al., 1996). Other study indicated that Phellinus linteus could protect primary cultured rat hepatocytes against hepatotoxins (Kim et al., 2004). In the present study, the increased levels of enzymes were considerably reduced by pre-treatment with ethanol extracts of PM. These results suggested that PM tended to prevent liver damage and suppressed the leakage of s-GOT and s-GPT through cellular membranes.

Histopathological examination of liver sections challenged with CCL₄ showed ballooning degeneration and inflammatory infiltration of lymphocytes (Gao et al., 2005). The liver sections of rats pretreated with the extract of Phellinus rimosus (Berk) showed well-preserved architecture (Ajith and Janardhanan, 2002). In our study, pretreatment with PM reduced the severity of the above mentioned histopathological injuries in CCL₄-intoxicated rats.

Many studies have shown that the hepatoprotective activities may be associated with an antioxidant capacity to scavenge reactive oxygen species (ROS) (Purmova and Opletal, 1995). ROS reacts with biological membrane and develops pathological situation (Vuillaume, 1987; Hemnani and Parihar, 1998). A potential mechanism of oxidative damage is the nitration of tyrosine residues of proteins, peroxidation of lipids, degradation of DNA, and oligonucleosominal fragments (Hemnani and Parihar, 1998). Antioxidant enzymes (SOD, catalase and GPx) offer protection against oxidative tissue damage (Halliwell and Gutteridge, 1990). Wang et al. demonstrated that sacrifice after 24 hours of CCL₄ administration (CCl₄/olive oil = 1:1, 3 ml/kg, sc) led to the increases in the activities of antioxidant enzymes including GPx, SOD, and catalase in the liver (2004). CCL₄ may cause oxidative stress and the consequent up-regulation of antioxidant enzymes and render cells more resistant to subsequent oxidative damage (Haliwell, 2000). In this work, PM reduced the increased antioxidant enzymes levels, implying that PM may prevent the peroxidation of lipids induced by CCL₄. PM protected against the injury of CCL₄ that may result from the interference with CYPs in a dose dependent manner. Pretreatment with PM extracts brought down the elevated levels of GPx, SOD and catalase. These biochemical restorations may be due to the inhibitory effects on CYPs and/or promotion of its glucuronidation (Wesley et al., 1992).

The methanolic extract of the basidiocarps of Phellinus linteus exhibits antioxidant effects (Chung et al., 1998). Phellinus rimosus (Berk) Pilat also possess antioxidant and antihepatotoxic activities (Ajith and Janardhanan, 2002). Phellinus linteus could protect primary culture of the rat hepatocytes against hepatotoxins (Kim et al., 2004). In our laboratory, we found that PM displayed antioxidant activities in comparison with chemicals in a series of in vitro tests (unpublished data). PM contains phenolic compounds. Hispolon, a phenolic compound from PM, exhibited antioxidant activity. Phenolic compounds possess antioxidant, radical scavenging, anti-mutagenic and anti-carcinogenic properties (Jayaprakasha et al., 2007). These results suggested that hispolon may be the active constituent of PM.
In conclusion, PM possessed hepatoprotective effect. The major cellular mechanism of hepatoprotective effect of PM may be related to increases in the activities of SOD, catalase and GPx. The active constituents of PM will be investigated in the future.

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References

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